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APPLICATION NUMBER: *60/457,570*
FILING DATE: *March 27, 2003*
RELATED PCT APPLICATION NUMBER: *PCT/US04/09662*

By Authority of the
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60457570 .032703

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This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No.

J1129 U.S. PTO 03/27/03

1011 U.S. PTO 60/457570

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☐ *Additional inventors are being named on the _____ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

LUPUS ANTIBODIES FOR PASSIVE IMMUNOTHERAPY OF HIV/AIDS

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☑ Specification *Number of Pages* 11 ☐ CD(s), Number

☐ Drawing(s) *Number of Sheets* ☑ Other (specify) Attachment to Specification (37 pages)

☐ Application Data Sheet. See 37 CFR 1.76

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Respectfully submitted,

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Date 03/27/2003

REGISTRATION NO. *(if appropriate)* 33,893

Docket Number: 330199.00100

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

Provisional Application

Lupus Antibodies for Passive Immunotherapy of HIV/AIDS

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Introduction

This invention describes the preparation of broadly reactive antibodies that bind to a conserved determinant of gp120 for the purpose of immunotherapy of HIV and immunoprophylaxis against HIV.

Recombinant antibody fragments derived from lupus patients who are not infected with HIV neutralize diverse strains of HIV belonging to clades B, C and D. These antibody fragments recognize a comparatively conserved determinant of HIV that is a constituent of the binding site of HIV coat protein gp120 for host cell CD4 receptors. The antibody fragments which neutralize HIV include single chain Fv constructs (VL and VH domains linked via a peptide linker) and light chain subunits. Intact antibody molecules that retain the properties of the Fv constructs can be readily isolated by methods well known in the art, such as (e.g., US patents 6,407,213 and 5,807,715). Improvements in the activity can be achieved by mutagenesis of the antibody genes and identification of the mutant proteins with the highest HIV binding and neutralizing activity (e.g, US patents 5,811,238 and 6,406,863).

The antibody fragments of this invention were isolated by phage display and selection for binding to the desired target antigen. This provisional application includes the attached manuscript entitled *HIV-1 Neutralizing Antibody Fragments to a Conserved Envelope Determinant from* Lupus Libraries, Karle *et al.)* and describes the phage display technique. Other, more routine methods of antibody isolation also allow isolation of the desired antibodies.

The lupus antibodies fragments are monovalent, and they display HIV neutralizing potency comparable to the best monovalent anti-HIV antibodies described in the literature. Recloning of

the monovalent fragments as bi- and multivalent fragments allows increased neutralizing potency due to avidity effects, as described for other antibodies in the literature. The human origin of these antibodies is a favorable factor, as this obviates anti-antibody responses.

The present antibodies and fragments are useful for passive immunotherapy in HIV-1 infected patients. Immunoprophylactic applications such as inclusion of the antibodies in a contraceptive cream are possible. Literature describing the use of antibodies in passive immunotherapy of HIV/AIDS are U.S. Patents 5,445,960; 5,695,927; and 5,783,670, incorporated herein by reference in their entirety. Additional patents describing standard methods for producing and using antibodies (e.g., b12 and X5) (Burton, Parren and Barbas from Scripps Institute, San Diego) and certain antibodies derived from hybridoma technology (Susan Zolla-Pazner group), are also incorporated herein by reference in their entirety.

This invention permits potent neutralization of HIV-1 across several clades. The neutralizing potencies are comparable or better than published values for other monovalent anti-HIV antibodies. In addition to the lupus clones described in the enclosed manuscript, additional Fv clones have recently been identified that display HIV neutralizing activity at concentrations as low as 0.025 microgram/ml (clones Fv JL 363 and Fv JL 379). These clones were isolated and analyzed for gp120 binding and HIV neutralization essentially as described in detail for Fv JL413 and JL427 here.

Lupus and HIV. Expression of retroviral genes which have migrated to the human genome has been implicated in the etiology of autoimmune disease (98). Antibodies to gp120 are found in lupus patients and mouse models of lupus (99-101). Epidemiological studies have noted the rare coexistence of lupus and AIDS (102-104). These considerations impelled us to isolate recombinant Ab fragments capable of binding gp120 from lupus phage display libraries for study of HIV neutralizing characteristics. Using selection and immunochemical procedures designed to isolate antibodies that recognize the gp120 determinant composed of residues 421-436, we succeeded in isolating several Ab fragments that neutralize each of the primary isolates of HIV-1 analyzed in PBMC cultures, belonging to clades B, C and D. Neutralization potency of the lupus

Ab fragments compare favorably with Abs proposed in the literature as promising immunotherapy candidates (5,6).

The Ab fragments are monovalent (Fv/L chains). Recloning these molecules as bivalent IgG improves neutralization potency even further due to avidity effects. Replacement mutations were clustered in the CDRs (compared to the FRs), which is a hallmark of affinity matured V genes encoding high affinity Ag binding antibodies. Thus, the lupus Ab repertoire is a source of neutralizing Abs to HIV-1.

Previously, we observed that polyclonal IgG preparations from autoimmune MRL/lpr mice (unimmunized) cleaved 125Igp120 (SF2 strain). Similarly, L chains isolated from polyclonal human lupus patients cleaved gp120, but a similar activity was not found in HIV-1 positive patients known to express gp120 binding Abs. One of 29 monoclonal L chain isolated from multiple myeloma patients was observed to cleave gp120 (31). Whether gp120 itself or a gp120-like retroviral Ag is the immunogen responsible for stimulating the formation of these Abs remains conjectural. The alternative, is that a self-Ag with homology to gp120 is the inciting immunogen. Homologies between certain self-Ags (HLA class I heavy chains, VIP and neurolikin) are known (105-107), but these homologies do not fall within gp120 residues 431-436.

Regardless of the inciting immunogen, the lupus anti-gp120 Abs ought to be valuable in HIV therapy. Therefore, we subjected the Fv and L chain phage libraries to covalent selection on the gp120(421-436)-CRA. Abs capable of cleaving monomer gp120 have also been isolated successfully by this strategy, some of which are potent inhibitors of HIV infection. Further studies are needed to determine the role of catalysis in the mechanism of neutralization. For instance, it is necessary to assess cleavage of native trimeric gp120 (as opposed to monomer gp120) to obtain useful correlations with HIV neutralization.

Lupus anti-HIV Ab fragments. Abs that bind the comparatively conserved epitope of gp120 composed of residues 421-436 are found in lupus (99,110). Amino acids within this determinant contribute important contacts in binding of HIV-1 by host cell CD4 receptors. Lupus Abs appear to recognize native trimeric gp120, indicated by observations of immunofluorescent staining of HIV infected cells with IgG purified from aged mice with lupus (MRL/lpr strain, these mice have a deficient Fas receptor gene; the resultant defect in lymphocyte apoptosis produces lupus-like autoimmune disease). The anti-gp120(421-436) binding Abs appear in the serum of the MRL/lpr mice as a function of age, concomitant with the onset of symptoms of autoimmune disease.

Human and murine lupus are associated with what appears to be an overall tendency towards synthesis of Abs with catalytic activity. Study of polyclonal lupus IgG indicated the presence of gp120-cleaving Abs. IgG samples purified by affinity chromatography from 17 HIV-1 positive patients showed little or no cleavage of 125I-gp120 by HIV IgG samples, but the 3 of 10 lupus IgG samples cleaved the protein, evident as depletion of the intact gp120 band and formation of smaller radioactive bands in SDS-gels. In separate experiments, L chains were purified from one of the gp120 cleaving lupus IgG samples (code 530) and from the serum IgG of lupus MRL/lpr mice (by reduction/alkylation of the IgG and FPLC gel filtration using previously described protocols, (89)). The 125I-gp120 cleaving activity was evident in the fractions corresponding to the L chain peak from both the lupus patient and the MRL/lpr mice (25 kD) (110).

The identity of the L chains recovered from the FPLC column was confirmed by SDS-PAGE and immunoblotting as described previously. In the presence of a serine protease inhibitor (0.3 mM DFP), gp120 cleavage by IgG from a lupus patient was essentially completely inhibited. In comparison, inhibitors of metalloproteases, cysteine proteases and acid proteases (EDTA, iodoacetamide, Pepstatin A) were without effect. Recombinant Fv and L chains clones capable of binding gp120 noncovalently and catalyzing its cleavage were isolated from human lupus libraries by various strategies: ***(i)*** Fv phage noncovalent binding to gp120 conjugated to Affigel with pH2.7 elution (40); ***(ii)*** Fv phage covalent binding to whole biotinylated gp120-CRA VIb followed by separation of the bound phage on an anti-gp120 column; ***(iii)*** L chain

phage noncovalent binding to immobilized gp120(421-436); and (iv) L chain phage covalent binding to immobilized gp120(421-436)-CRA Vc followed by recovery of bound phages by reduction of the S-S bond placed located in this CRA (45). Soluble Fv fragments were obtained by expression of the selected phagemid DNA (periplasm of HB2151 cells; this bacterial host reads a codon located between the Ab insert and the p3 protein as a stop codon, allowing soluble expression of the recombinant proteins). These were analyzed for: ***(i)*** binding to full-length gp120 (monomeric) and synthetic gp120(421-436) conjugated to albumin; ***(ii)*** cleavage of monomer gp120, ***(iii)*** neutralization of HIV-1. Noteworthy results are: **(a) Following phage noncovalent selections,** increased binding of gp120 binding and gp120(421-436) binding was evident in the Fv and L chain populations, indicating the success of selection procedures. With the exception of one clone, binding of gp120 and gp120(421-436) were correlated. Dose-dependent antigen binding by the purified Ab fragments was observed. Binding of one of the clones to irrelevant proteins (calmodulin, thyroglobulin, albumin) was analyzed. No significant recognition of these proteins was observed. Two important conclusions may be drawn. First, determinant 421-436 in the synthetic peptide and full-length gp120 must share substantial structural similarity. Second, most gp120 binding Abs in lupus are directed at recognition of the gp120(421-436) epitope. Immunogenetic analysis of lupus Abs has previously shown that some of these do not contain sufficiently diversified V domain sequences to anticipate Ag-specific binding activities (such Abs usually display polyreactive binding patterns). This was not the case for the gp120-binding Abs. Sequence analysis of the two Fv clones and one L chain indicated the presence of extensive replacement mutations compared to their germline V gene counterparts (Table 2). The mutations tended to cluster in the complementarity determining regions (versus framework region of the V domains), and the ratio of replacement to silent mutation was generallygreater for the CDRs than the FRs. This is a sign that the Ab V domains have been subjected to adaptive sequence diversification typical of B cell clonal selection. The purified Fv and L chain clones isolated by noncovalent binding techniques neutralized primary isolates of HIV-1 with impressive potency. This was evident from measurement of infection of peripheral blood mononuclear cells (PBMC) by three primary isolates of HIV-1belonging to clades B, C and D. Inhibition of infection was not due to a cytotoxic effect, as no loss of cell viability was observed following incubation with Ab fragments in the absence of HIV (determined by staining

cells with acridine orange/ethidium bromide; Sigma; viability ~80-85% in cells treated with diluent and the Ab preparations). No neutralization of HIV-1 was observed in the presence of equivalently purified irrelevant control Fv and L chain clones or purified extracts of bacteria harboring vector devoid of Ab inserts were also devoid of inhibitory activity. IC50 for Fv clone 683 is 6 ng/ml (strain ZA009). This compares favorably with the neutralization potency of candidate immunotherapeutic Abs described in the literature (e.g., Fab fragments of Ab b12; other bivalent IgG molecules isolated from HIV+ subjects or derived from in vitro molecular evolution strategies. *(b)* Two types of covalent phage selections indicate the feasibility of HIV-1 targeting by specific gp120-cleaving Abs from the lupus repertoire. First, gp120 peptide-CRA Vc was applied for selecting phage L chains. Phage-CRA complexes formed by mixing these reagents were trapped on a streptavidin column and the phage subpopulation complexed irreversibly to the CRA (i.e., phage subpopulation not removed by pH2.7 and pH11 buffer washes) were recovered by cleaving the S-S bond located between the biotin and the phosphonate moiety. Initial catalysis assays utilized the substrate gp120(421-432)-methylcoumarinamide (MCA; the targeted bond is K432-MCA, located at the position of the phosphonate moiety in CRA Vc). Measurement of aminomethylcoumarin release (by fluorimetry as in our previous studies), suggested a catalytic activity in the recovered clones.

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Claims

1. Antibodies or antibody fragments that neutralize HIV-1 derived from patients with autoimmune disease.

2. Antibodies or antibody fragments that neutralize HIV-1, derived from patients with lupus.

3. The antibodies of claim 1 or 2, which are monoclonal antibody fragments obtained by cloning cDNA for the antibody variable domains of the light chain (VL) and heavy chain (VH) from mRNA expressed by lymphoid cells.

4. The monoclonal antibody fragments of claim 3 obtained as single chain Fv constructs comprising the VL and VH domains linked by a flexible peptide linker.

5. The antibody fragments of claim 4 in which the order of arrangement of the components from N terminus to C terminus is VL-linker-VH

6. The antibody fragments of claim 4 in which the order of arrangement of the components from N terminus to C terminus is VH-linker-VL.

7. The antibody fragments of claim 4 in which the amino acid constitution of the linker is varied by mutagenesis to optimize the VL-VH interface supporting the greatest HIV neutralizing activity.

8. The antibody fragments of claim 4 in which the length of the linker is varied by mutagenesis to optimize the VL-VH interface supporting the greatest HIV neutralizing activity.

9. Monoclonal antibody light chain subunits of claim 1, obtained by cloning the cDNA for VL and light chain constant regions (CL).

10. Monoclonal Fv constructs of claim 4 reactive with HIV, obtained by expression of the Fv constructs on the surface of phage particles and identifying the subpopulation of HIV-reactive Fv particles by binding to intact HIV, trimeric gp120, monomer full-

length gp120 and synthetic peptide fragments of gp120 .

11. The monoclonal Fv constructs of claim 10 which neutralizes at least three strains belonging to different HIV clades.

12. The monoclonal Fv constructs of claim 10, which neutralize strains belonging to HIV-1 clades B, C and D.

13. Monoclonal full-length immunoglobulins obtained by recloning Fv fragments of claim 11 as IgG, IgA or IgM constructs.

14. Monoclonal light chains of claim 9 reactive with HIV, obtained by expression of the light chain on the surface of phage particles and identifying the subpopulation of HIV-reactive light chain particles by binding to intact HIV, trimeric gp120, monomer full-length gp120 or synthetic peptide fragments of gp120 .

15. Monoclonal light chains of claim 14, which neutralize at least three strains belonging to different HIV clades.

16. Monoclonal light chains of claim 14, which neutralize strains belonging to HIV-1 clades B, C and D.

17. Monoclonal Fv constructs containing the VL domains from light chains of claim 15 and 16 paired with the VH domain of other anti-gp120 antibodies.

18. Monoclonal full-length immunoglobulins obtained by recloning Fv fragments of claim 17 as IgG, IgA and IgM constructs.

19. Monoclonal full-length immunoglobulins with binding specificity for HIV obtained by preparing a plurality of hybridomas of lymphoid cells from lupus patients and myeloma cells and screening said hybridomas for secretion of antibodies for HIV binding capability and HIV neutralizing activity.

20. Monoclonal full-length immunoglobulins of claims 13, 18 and 19, which neutralized

at least three strains belonging to different HIV clades and subtypes.

VERSION 03-18-03

HIV-1 Neutralizing Antibody Fragments to a Conserved Envelope Determinant from Lupus Libraries

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Running title: Lupus anti-HIV antibodies

ABSTRACT

Cross-clade neutralizing antibodies (Abs) to conserved HIV-1 components are highly sought because of their potential use in immunotherapy of AIDS. HIV-1 infection occurs only rarely in lupus patients. We hypothesized that intrinsic immunological responses in lupus patients protect against HIV infection. Phage displayed single chain Fv and light chain libraries from lupus patients were selected and screened by procedures allowing isolation of clones capable of binding coat protein gp120 residues 421-436, a comparatively conserved component of the CD4 binding site. Binding of synthetic gp120(421-436) and full-length gp120 by the Ab fragments was correlated, indicating the peptide determinant to be the major target of the Abs. Mutations were clustered in the complementarity determining regions, suggesting that the Ab variable domains are products of adaptive affinity maturational processes. Infection of peripheral blood mononuclear cells by four diverse HIV-1 primary isolates was neutralized by the Fv fragments. The functional properties of the Fv clones point to the lupus repertoire as a unique source of Abs with possible utility in HIV immunotherapy.

Abbreviations. Ab, antibody; BSA, bovine serum albumin; CD4bs, CD4 binding site; PBMC, peripheral blood mononuclear cells

Identifying broadly neutralizing Abs to the envelope proteins of HIV has been a long-standing goal. Such Abs can help determine which antigenic determinants are suitable as targets for prophylactic vaccination. Moreover, the Abs themselves could potentially be applied for passive immunotherapy of AIDS if derived from a human source and available in monoclonal or recombinant form. Studies of the immune responses mounted in HIV-infected subjects and induced by experimental immunization with the envelope protein gp120 have indicated that the main epitopes recognized by Abs are the variable domains of the protein (*1*). These Abs generally do not neutralize primary HIV-1 isolates. Moreover, as the Ab-targeted epitopes are highly mutable, the Abs do not neutralize diverse HIV-1 strains found in different geographical regions and HIV-1 mutants that evolve in the course of infection in the same individual. Discontinuous peptide determinants constituting the site in gp120 responsible for recognizing host cell CD4 receptors, the CD4 binding site (CD4bs), tend to be conserved in diverse HIV-1 isolates. Abs to the CD4bs are formed only rarely, but certain Abs that recognize the CD4bs of monomeric gp120 have been prepared from sources such Epstein Barr virus-transformed lymphocytes of HIV-1 infected individuals (*2*), Ab fragment libraries from an HIV-1 infected individual who remained free of AIDS for an extended period (*3,4*), and mice immunized with gp120 (*5*). Disappointingly, only a minority of the known Abs to the CD4bs display cross-clade neutralizing activity, a finding that has recently been attributed to conformational transitions occurring when native trimeric gp120 expressed on the viral surface dissociates into its monomeric form (*6*).

Systemic lupus erythematosus, an autoimmune disease, and HIV/AIDS, an immunodeficiency disease, coexist rarely. Although controlled epidemiological studies weighted for sexual habits and intravenous drug use remain to be carried out, several reports have discussed the strikingly small number of patients with concomitant lupus and HIV-1 infection, only twenty-nine cases worldwide (*7-10*). Interestingly, Abs to HIV-1 have been identified in the serum of lupus patients and mouse models of lupus (*11-14*). As there is no evidence of HIV-1 infection in the Ab-positive lupus subjects, the mechanism of Ab formation and their functional significance remains enigmatic. If the lupus repertoire expresses Abs to conserved gp120 determinants, it can serve as a useful source of anti-HIV Abs. These considerations impelled us to study M13 phage-

displayed Ab libraries prepared from the peripheral blood lymphocytes of HIV-negative lupus patients (*15*) as the source of HIV neutralizing Abs. We report the identification of affinity matured single chain Fv and light chain subunits capable of recognizing a conserved peptide determinant of gp120, residues 421-436. This determinant is a component of the CD4bs *(16,17)*. The Fv clones neutralized the infectivity of diverse HIV-1 strains, raising interesting possibilities regarding their functional role and utility as anti-HIV reagents.

Materials and Methods

Lupus Fv and L chain clones

Preparation of the following phage displayed libraries has been described previously (*15*): ***(a)*** human L chains cloned in pCANTAB5his6 phagemid vector (pooled light chain cDNA from 3 lupus patients); ***(b)*** human single chain Fv constructs in pHEN2 phagemid vector (V_L-linker-V_H; linker, SS[GGGGS]$_2$GGSA; pooled Fv cDNA from 2 lupus patients). Phages displaying the Fv libraries as p3-fusion proteins were packaged from TG1 cells using M13K07 helper phages (10^{13} particles) and selected by column chromatography on recombinant gp120 (strain SF2: Austral Biologicals) immobilized on Affigel-10 (Biorad) via Lys sidechains (1 ml settled gel; 47 µg gp120/ml settled gel) using a pH 2.7 buffer for elution of bound phages (*18*). Similar procedures were applied to the L chain library except that the library had been preselected by binding to a phosphonate diester hapten (compound II in ref *15*; this step enriches antibodies with nucleophilic activity, see ref *15* discussion section). Phage selection was conducted by "panning" on synthetic gp120(421-436) (KQIINMWQEVGKAMYA, corresponding to the consensus sequence of this determinant in clade B strains; *19*). The peptide was immobilized (Nunc Maxisorp tubes; 10 µg peptide/tube), blocked with 5% BSA, incubated with phages for 1 h and unbound phages removed by washing with10 mM sodium phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4 containing 0.05% Tween-20 (PBS-Tween). Bound phages were eluted with 0.1 M glycine-HCl, pH 2.7 and immediately brought to neutral pH using 1 M Tris base (*20*). HB2151 cells were infected with the eluted phages, permitting expression of the antibody fragment in soluble form. Periplasmic extracts were prepared following induction with isopropyl-β-D-thiogalactoside and recombinant proteins were purified by metal affinity chromatography to

electrophoretic homogeneity (the Ab fragments contain a his_6 tag; *15)*. SDS-polyacrylamide was on 8-25% Phast gels (Pharmacia), with identity of the proteins confirmed by immunobloting using anti-cmyc Ab as described previously (*15;* the Ab fragments contain a ten residue c-myc peptide close to their C terminus).

ELISA

Immobilized Cys-gp120(421-436) conjugated to bovine serum albumin (BSA; 10 mol peptide/mol BSA; 230 ng peptide equivalents/well) or full-length monomer gp120 (100 ng/well , MN strain, Immunodiagnostics Inc.) were used for ELISA (*19*). These were coated on Maxisorp 96-well microtiter plates (Nunc; 1 h), the plates blocked with 5% skim milk and incubated with recombinant Ab fragments in PBS-Tween containing 0.1% skimmed milk (1 h) in triplicate wells. Bound Ab fragments were detected using mouse anti-c-myc Ab (clone 9E10; 1:500 delipidated ascites) followed by peroxidase-conjugated goat anti-mouse Fc specific IgG (1:1000; Sigma) for color development. In competition ELISAs, the recombinant Abs were pretreated with diluent or competitor proteins (1 µM; calmodulin, BSA, thyroglobulin; Sigma) for 1 h and then analyzed by ELISA.

HIV-1 neutralization

HIV neutralization assays using PBMC hosts were carried out as described in (*21,22*) but with p24 quantification as the measure of infection. The following primary isolates of HIV-1 were obtained from the NIH AIDS Research and Reference Reagent Program: ZA009 (coreceptor CCR5, clade C), BR004 (coreceptor CCR5, clade C), Ug046 (coreceptor CXCR4, clade D) and SF-162 (coreceptor CCR5, clade B). HIV-1 primary isolate strain 23135 (coreceptor not known, clade B) was from Dr. Sandra Levine (Univ Southern California). Each virus stock was titered in preliminary studies with each batch of donor PBMCs to determine the working dilution giving the optimum TCID50. The working dilution was adjusted to give p24 signal sufficient to be measured reproducibly in the linear range of the p24 assay after 4 days. The virus in RPMI was treated in quadruplicate with equal volumes of increasing concentrations (up to 50 µg/ml) of metal affinity-purified Fv or L chain in PBS (1 h; TCID50 for virus = 100). PHA-stimulated PBMCs from healthy human donors (0.25 million) were added to virus-Ab fragment mixtures and incubated for 3 days

(37°C), the cells washed twice with PBS and once with RPMI1640 (this removes potential interfering p24 from the original inoculum), incubated in fresh RPMI for 24 h, lyzed with Triton X-100, and p24 in supernatants measured by an enzymeimmunoassay kit (Beckman Coulter HIV-1 p24 Antigen Assay Kit; linear range 50-3200 pg/ml). PBMC viability determination following incubation with Fv JL413, Fv JL427 or L chain SK18 (27 µg/ml, 72 h; incubations done exactly as in virus neutralization assays) was by staining with a mixture of acridine orange (2 µg/ml) and ethidium bromide (1 µg/ml) following by counting the viable cells (green fluorescence) using a hemocytometer and a UV microscope (23). Negative controls included the virus (strain ZA009) treated with: (a) diluent, (b) metal affinity purified extract of bacteria harboring pHEN2 vector (processed identically as recombinant Ab preparations); (c) light chain clone GG63 and SK161 (11 µg/ml, ref *15*); and (d) Fv clones JL610 and JL611 (2.5 µg/ml). IgG clone b12 was kindly provided by Dr. Dennis Burton as a reference Ab.

Results

Lupus anti-gp120 antibody fragments

Single chain Fv constructs similar to those studied here reproduce faithfully the binding activity of full-length IgG Abs (e.g., *24*). Previous reports have documented the antigen binding activity of L chain subunit independent of its H chain partner, albeit at reduced strengths compared to native Abs composed of both subunits (*25,26*). In the present study, two types of phage selections were carried out to enrich anti-HIV Ab fragments present in the lupus libraries: binding of Fv phages by full-length gp120, and of L chain phages by synthetic gp120(421-436). Monoclonal soluble Ab fragments obtained by expression of the selected phagemid DNA in HB2151 bacteria were screened for binding to gp120 and gp120(421-436) by ELISA. Fifty four percent and 26% of selected Fv and selected L chain clones were bound by full-length gp120, respectively, and 31% and 17% were bound by gp120(421-436) (Fig 1A). Except in the case of one Fv clone, binding of the two antigens was highly correlated (Fig 1B).

Two Fv clones (JL413, JL427) and one L chain clone (SK18, GenBank accession pending) were characterized further. Electrophoretically pure Fv and L chains from these clones displayed concentration-dependent gp120 and gp120(421-436) binding (Fig 2A-C). Competitive ELISA

studies indicated no significant reactivity of Fv JL427 with proteins unrelated to gp120(421-436) (Fig 2D).

Replacement mutations located in the CDRs of the V genes are usually interpreted to indicate adaptive maturation via somatic hypermutation processes (27). Comparison of the cDNA sequences of Fv JL413 and JL427 with their closest germline V gene counterparts revealed extensive replacement mutations in the regions contributed by the V_L and V_H genes (Table I; excluding the regions encoded by PCR primers or contributed by the D/J genes). The mutations tended to cluster in the complementarity determining regions. The ratios of replacement to silent mutations in the six CDRs for each Fv clone was greater than for the FRs. The V_L domain of L chain clone SK18 contained 4 replacement mutations, with 1 replacement in the CDRs. Nonetheless, the replacement/silent mutation ratio for the CDRs remains greater than for FRs, as all of the silent mutations are located in the FRs. Additional alignments of the Fv and L chain clones with the closest germline V, (D) and J genes were attempted to assess junctional diversification. with introduction of gaps due to deletions and exclusion of FR4 sequence differences generated by the forward PCR primer [see Table 1 for germline J and D usage information; D gene usage for Fv JL427 V_H is uncertain because of lack of discernable identity with germline D genes; the shortest available D segment, (D7-27, 11 nucleotides) was selected for alignment to obtain the most conservative diversification estimates]. The sequence analyses suggested extensive deletion and replacements as follows (number and gene identity in parentheses): Fv JL413 V_L domain, 3 deletions (2V, 1J); Fv JL413 V_H domain, 17 deletions (7V, 5D, 5J), 3 replacements (3D); Fv JL427 V_L domain, 1 deletion (1V), 1 replacement (1J); Fv JL427 V_H domain, 4 deletions (1V, 3D), 5 replacements (5J); L chain SK18 V_L domain, 2 deletions (1V, 1J), 1 replacement (1J).

Cross-clade HIV-1 neutralization

HIV-1 infection studies were done using PHA-stimulated peripheral blood mononuclear cells (PBMCs), with measurement of p24 antigen serving as the index of the level of infection (*21,22*). Progressively increasing neutralization of strain ZA009 by increasing concentrations of Fv JL413, Fv JL427 and light chain SK18 was observed (Fig 3). In control studies, no loss of HIV infectivity

(strain ZA009, clade C) was evident in the presence of identically purified Fv and L chains from several irrelevant clones (control Fv clone JL610 and L chain clone GG63 shown in Fig 3; not

shown, control Fv clone JL611, 2.5 µg/ml; control L chain clone SK161, 11 µg/ml) and the purified extract of bacteria harboring vector devoid of antibody inserts (*15*). In each case, p24 levels were statistically indistinguishable from virus control wells treated with PBS ($p > 0.05$, Student's t-test). At the aforementioned concentrations of the control clones, >75% neutralization of ZA009 strain was consistently observed in the presence of Fv JL413, Fv JL427 and L chain SK18. Inhibition of infection was not due to a cytotoxic effect, as no loss of cell viability was observed following incubation with Ab fragments in the absence of HIV (*23*). PBMC viability after treatment with diluent or the Ab fragments (Fv JL413, Fv JL427 or L chain SK18, 27 µg/ml) was 81.2 ± 2.8% and 74.8 – 80.9%, respectively (150-200 cells counted). Consistent with the conserved nature of the antigenic recognition site (residues 421-436 of gp120), dose-dependent neutralization of each of the primary HIV-1 isolates by the purified Fv clones was observed (Table II). The infectivity of strains drawn from three distinct clades (B, C and D) was inhibited by the Fv constructs, suggesting broadly reactive neutralizing activity. Strains ZA009, SF-162 and BR004 utilize coreceptor CCR5, and clade D strain Ug046, coreceptor CXCR4. The L chain clone neutralized two of the three strains analyzed. Assays using independent preparations of the Ab fragments reproducibly indicated dose-dependent neutralization [strain ZA009, N=3, IC50 for L chain SK18 L chain, Fv JL413 Fv and Fv JL427 in µg/ml: 0.4 ± 0.3, 0.2 ± 0.1 and 0.3 ± 0.1 (s.d.), respectively; strain Ug046, N=2, IC50 for L chain SK18 and Fv JL413: 11.6-13.5 and 2.1-5.5 µg/ml, respectively]. Side-by-side comparisons suggested that the neutralizing potency of monovalent Fv JL413 was comparable to bivalent IgG b12, a broadly neutralizing Ab to the CD4bs (*28*) (Fig 3).

The utility of binding to synthetic gp120(421-436) as a means for identifying HIV-neutralizing antibody fragments from the lupus repertoire is evident from these results. However, the binding data do not fully predict neutralization efficacy. For instance, L chain clone SK18 bound the antigens at considerably lower levels than the Fv clones (>2.7-fold for gp120(421-436) and >3.0-fold for gp120, Fig 2), but strains ZA009 and Ug046 were neutralized with similar potencies by all three Ab fragments (Table II). Differences in strain-reactivity of the individual Ab fragments are also evident. This is best exemplified by the L chain – no neutralization of strain 23135 was observed, whereas strains ZA009 and Ug046 were neutralized potently.

Discussion

The lupus Fv clones described here bind a comparatively conserved constituent of the CD4bs, i.e., residues 421-436, and they display potent, cross-clade neutralization of primary HIV isolates dependent on R5 and X4 coreceptors. Polyclonal Abs to synthetic gp120(421-436) have been described previously in the serum of lupus patients and MRL/lpr mice, which spontaneously develop lupus-like symptoms (*11,14*). As the Fv clones were selected by binding to full-length gp120, their ability to recognize gp120(421-436) suggests this determinant to be the major epitope recognized by lupus Abs. Conversely, as the L chain clones were originally selected by binding to gp120(421-436), their reactivity with full-length gp120 indicates the ability of the synthetic peptide to assume a conformation similar to the cognate determinant expressed in full-length gp120. Promiscuous antigen binding activity patterns by Abs containing minimally mutated V domains have been described in previous studies (e.g.; *29*). These properties contrast with the gp120 binding Fv clones described here, in that extensive mutations were identified in the V domains and the binding activity was specific for gp120. Broad implications of the data reported here are: ***(a)*** The Abs may help dissect the antigenic structure of the CD4bs to identify important components susceptible to protective humoral immune response; ***(b)*** If the Abs fulfil certain additional criteria regarding the breadth, potency and ability to neutralize HIV-1 infection *in vivo*, they may serve as prototypes for the development of immunotherapeutic Abs; and ***(c)*** The detection of HIV neutralizing Fv clones in the library taken together with previous serum Ab studies (*11,14*) offers a rational explanation for the infrequent incidence of AIDS in lupus patients.

Structural analyses have suggested that certain contact residues in the complex of gp120 and CD4 are contributed by residues 421-436 (*16,17*). These residues are comparatively conserved in different HIV strains, presumably because CD4 binding is essential to maintain viral infectivity. Sequence polymorphisms in determinant 421-436 are located primarily at residues 429 and 432 (assessed by comparing all available sequences of this determinant in the Los Alamos database; 384 HIV-1 strains, clades A, B, C, D, F, G, H, J, U, N and O as well as the CRF and CPZ classifications; 40.7% and 42.0% of the strains contain the consensus residues at these positions; remaining positions are occupied by the consensus residues in 82.7 – 99.5% of the

strains). Fine structural differences in the CD4bs may result from the sequence polymorphisms with important consequences in regard to neutralizing activity. Full assessment of the extent to which the neutralizing activity of lupus Abs is tolerant to sequence divergence requires study of a large panel of HIV isolates containing defined polymorphisms in residues 421-436. Some initial conclusions are possible, however. The two Fv clones effectively neutralized strains ZA009 as well as Ug046 despite the differing identity of the residue at position 429 (Lys and Gly, respectively). Similarly, binding of the Ab fragments to synthetic peptide determinant 421-436 derived from strain SF2 was generally correlated with that to full-length gp120 from strain MN, despite the sequence difference at position 429 (Glu and Lys, respectively). Evidently, the Ab binding and neutralizing activities can be maintained despite certain sequence polymorphisms.

In addition to cross-clade HIV-1 neutralizing activity, high level potency is desirable for consideration of Abs in immunotherapy applications. Neutralizing potencies of the Fv clones compare favorably with the monovalent Fab fragments and bivalent IgG Abs proposed as candidates for HIV immunotherapy (e.g., *4*). Further gains may be realized by recloning monovalent lupus Fv clones as bivalent IgG. The neutralizing potency of a monovalent Fab b12 is reportedly increased by 400-fold by recloning as a bivalent IgG, presumably due to increased avidity effects (*28*). Ab catalyzed gp120 digestion is another potential route towards enhanced potency, particularly in regard to the L chain subunit. Ab fragments with the ability to cleave peptide bond surrogates have been identified in the lupus phage libraries utilized in the present study (*15*) and two Ab L chains from other sources have previously been shown to cleave HIV coat proteins (*14,30,31*). Evidence that lupus Ab fragments express the correct epitope-specificity, i.e., recognition of determinant 421-436, suggests the feasibility of directing the catalytic function to this region of gp120. No cleavage of gp120 by the Ab fragments reported here was detected (assayed as in ref *14*; data not shown), but phage selection conducted with a covalently reactive peptide analog of determinant 421-436 (*31*) have permitted identification of a gp120 cleaving L chain (Paul, S. and coworkers, to be published elsewhere).

The ability to bind the linear synthetic determinant 421-436 sets lupus Ab fragments apart from CD4bs-directed Abs described previously, the antigen binding activity of which depends on recognition of discontinuous segments of the CD4bs. The broad viral neutralizing behavior of the

lupus Abs is another distinctive property. Based on the thermodynamic parameters for binding of various Abs, a recent report has deduced conformational differences between the trimeric CD4bs of gp120 on the viral surface and the corresponding site in monomer gp120 (*6*). This helps explain the lack of broad HIV neutralizing activity of Abs that bind the CD4bs of monomer gp120 (e.g., *2*). The functional properties of lupus Fv fragments compared to Abs formed in HIV-1 infection probably derive from unique immunological selection processes. The Fv mutational patterns (Table II) taken together with apparent absence of binding to unrelated proteins by Fv clone JL427 suggest that the lupus library can serve as a source of HIV-specific neutralizing Abs. It is difficult to attribute the HIV neutralization to cross-reactivity of anti-self Abs, as there are no known sequence identities between determinant 421-436 and human proteins, although other regions of gp120 express limited homologies with certain self antigens (*33,34*). We are left with the hypothesis that expression of endogenous retroviral gene products expressing structural similarity to the CD4bs may drive the synthesis of HIV Abs in lupus patients. The presence of retroviral gene sequences in the genome of uninfected humans is well known, and links between expression of such sequences and autoimmune disease have been discussed previously (*35,36*).

A previous study demonstrated the neutralization of a lab-adapted and a primary isolate of HIV-1 by polyclonal Abs present in sera of patients with mixed connective tissue disease and attributed the neutralizing activity to Abs that recognize the V3 loop of gp120 (*37*). Unfortunately, use of serum Abs precludes unambiguous assignment of the neutralizing activity to individual components in polyclonal mixtures. As rare Abs can be readily isolated by phage display methods, the present study does not allow inferences about the relative ability of lupus patients and non-autoimmune subjects to produce Abs directed to gp120(421-436). However, serum Abs capable of binding this determinant have been observed in human and murine lupus but not in control non-autoimmune subjects (*11, 14*), suggesting that production of these Abs is not a generalized phenomenon. These reports, taken together with the HIV-1 neutralizing activity of recombinant lupus Ab fragments in the present study, raise the possibility of a protective role of the Abs in defense against HIV. The presence of the Abs may help explain clinical observations of the rare coexistence of lupus and AIDS. Conversely, certain reports discuss clinical

amelioration of human lupus occurring upon the onset of viral immunodeficiency disease (*9*). In a murine experimental model, the symptoms of lupus are alleviated by induction of retroviral infection (*37*).

Acknowledgments

Supported by US Public Health Service grants AI46029 and AI31268 and a Texas Technology Development and Transfer grant. We thank John Marchalonis for reviewing the manuscript prior to submission.

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LEGENDS

Fig 1: Selection of phage anti-gp120 antibody fragments from lupus libraries (A); and, correlated binding of lupus antibody fragments to full-length gp120 and synthetic gp120(421-436) (B). Shown are ELISA values for Fv and L chain clones fractionated by prior binding of phage particles to immobilized gp120 and synthetic gp120, respectively (selected clones) or picked randomly from the unfractionated source libraries (unselected clones). N= number of independent clones analyzed. ***A, Top,*** Immobilized synthetic gp120(421-436) conjugated to bovine serum albumin. ***A, bottom,*** Immobilized full-length gp120. ***B,*** Plotted are selected Fv and L chain clones displaying A490 > 0.3 in Fig 1. Fv clones shown are: JL409, JL413, JL437 (●) and JL427 (■). L chain clones SK18, SK45, SK41, SK51 (▲). P = 0.0004 for regression line (computed by excluding Fv JL427; r = 0.24, P = 0.15 with inclusion of this Fv). Data are corrected for binding by periplasmic extracts of bacteria harboring the vector without antibody insert [pHEN2 vector; A490 0.10 and 0.14 for gp120(421-436) and gp120 binding, respectively]. Recombinant antibody expression determined for 10 clones was 1.95 ± 0.51 (s.e.m.) mg/liter bacterial culture. Differences in binding of gp120(421-436) and full-length gp120 by different clones observed by ELISA were not due to variations of the expression level (r^2 0.10 and 0.17, respectively, P>0.24). PCR amplification using antibody framework primers confirmed the presence Fv and L chain inserts in the phagemid DNA (~0.7 kb; ref *15*).

Fig 2. Concentration-dependent binding of immobilized gp120(421-436) (■) and full-length monomer gp120 (●) by lupus antibody fragment (A-C) and specificity of binding to immobilized gp120(421-436) (D). Recombinant proteins purified by metal afffinity chromatography (*13*). **D,** Fv JL427 (46 µg/ml) treated with equal volume of soluble gp120(421-436), bovine serum albumin (BSA), thyroglobulin (Tg) and calmodulin (CaM) competitors (1 µM). ***Insets,*** silver stained SDS-polyacrylamide electrophoresis gels (8-25%) showing 27 kD purified antibody fragments purified (right lane in each panel) and marker proteins (left lane; from top to bottom, 94, 67, 43, 30, 20, 14 kD; Pharmacia).

Fig 3. Concentration-dependent HIV-1 neutralization by lupus antibody fragments. A & B, HIV-1 strain ZA009 (clade C), host cells PBMCs from healthy human donors. **C,** HIV-1

strain BR004 (clade C), host cells PBMCs from healthy human donors. See Table II legend for method and curve fitting procedure. Values are percent of p24 concentrations in culture wells containing HIV treated with PBS instead of Abs. Clones L chain SK18, Fv JL413, Fv JL427 and IgG b12: 4 independent culture replicates analyzed individually for p24 concentration (means ± sem). Clones L chain GG61 and Fv JL610: pooled supernatants from 4 culture wells.

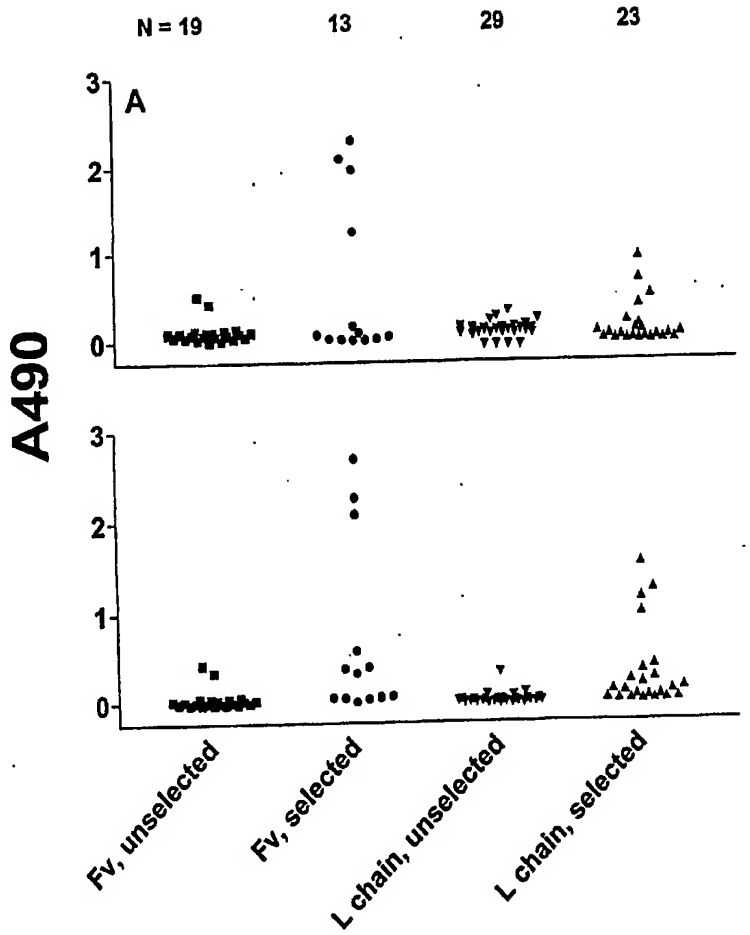
	L chain SK-18	Fv JL-413		Fv JL-427	
		VL	VH	VL	VH
Family	I	I	IV	I	V
Subgroup	I	I	II	I	III
Germline	02/012, Jk1	L5, Jk4	VH4-59, D2-15, JH5	V1-17, JL3	VH3-48, D*, JH6
Counterpart Mutations					
R	4	9	4	14	13
S	3	3	3	8	2
R/S CDRs	1/0	6/0	3/1	9/3	8/1
R/S FRs	3/3	3/3	1/2	5/5	5/1

Table I. Lupus antibody characteristics deduced from V domain sequences. R – Replacement; S – Silent; D* – germline D gene unassignable. Germline counterparts identified from http://www.ncbi.nlm.nih.gov/igblast (*39,40*). V gene mutation counts restricted to residues 8-95, V_L domain, L chain SK18 (linear numbering); 8-94 and 8-91, V_L and V_H domains of Fv JL413, respectively; 8-120 and 8-98, V_L and V_H domains, Fv JL427, respectively. These residues correspond to lengths of germline V genes identified in lupus clones. FR1 residues 1-7 excluded because these are encoded by PCR back primers. Family and subgroup assignment from http://immuno.bme.nwu.edu/. cDNA sequences of Ab V domains determined in the 5' and 3' directions were identical.

Ab	B, 23135		B, SF-162		C, ZA009		C, BR004		D, Ug046	
	IC50	IC90	IC50	IC90	IC50	IC90	IC50	IC90	IC50	IC90
SK18 L chain	>54	--	nt	nt	0.1	2.3 (0.7, 1.00)	nt	nt	13.5	18.8 (6.6, 1.00)
JL413 Fv	25.6	44.6 (4.2, 0.99)	2.2	97.7 (0.4, 0.99)	0.1	16.1 (0.5, 1.00)	0.1	2.8 (0.7, 0.98)	1.0	10.0 (1.0, 0.97)
JL427 Fv	22.4	36.7 (4.5, 0.97)	nt	nt	0.3	5.7 (0.7, 1.00)	nt	nt	5.5	12.7 (2.6, 0.96)
b12 IgG	nt	nt	1.1	14.1 (0.9, 0.94)	nt	nt	9.0	333 (0.6, 0.89)	nt	nt

Table II. HIV-1 neutralization by lupus Ab fragments. Values are reported as µg Ab fragment/ml yielding 50% (IC50) and 90% (IC90) neutralization. In parentheses are, respectively, Hill slopes and squared correlation coefficients for curves fitted to the equation: % HIV neutralization = $100\%/[1+10^{((\log IC50 - \text{Ab concentration}) \times \text{Hill slope})}]$. Curves forced through origin (zero neutralization); Hill slope value held as a variable parameter. nt, not tested.

Figure 1


N = 19
13
29
23
A
A490
3
2
1
0
Fv, unselected
Fv, selected
L chain, unselected
L chain, selected

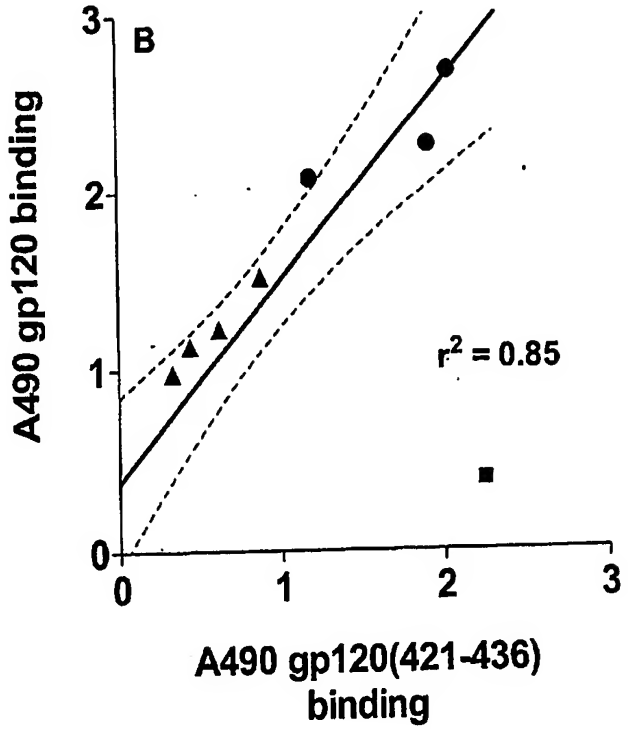

B
A490 gp120 binding
A490 gp120(421-436) binding
r2 = 0.85
0
1
2
3

Figure 2

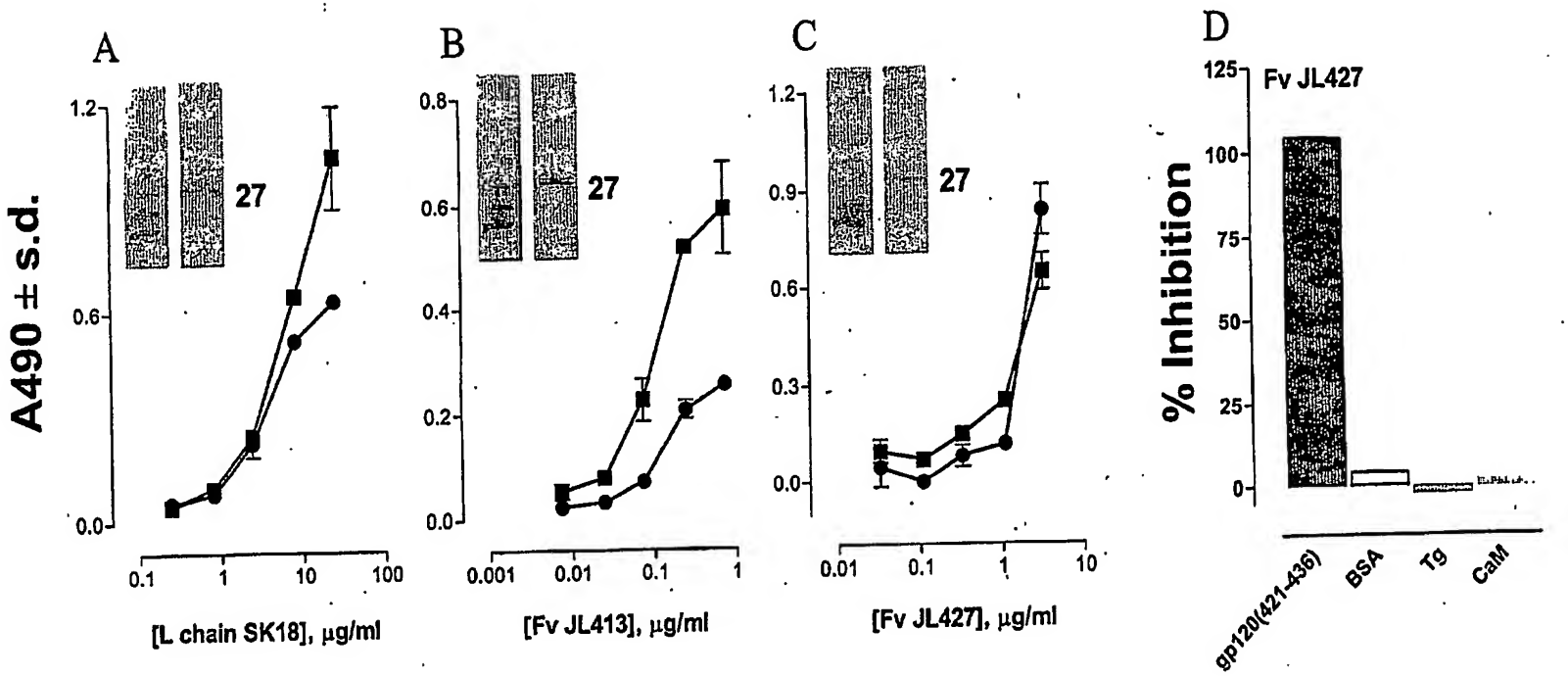
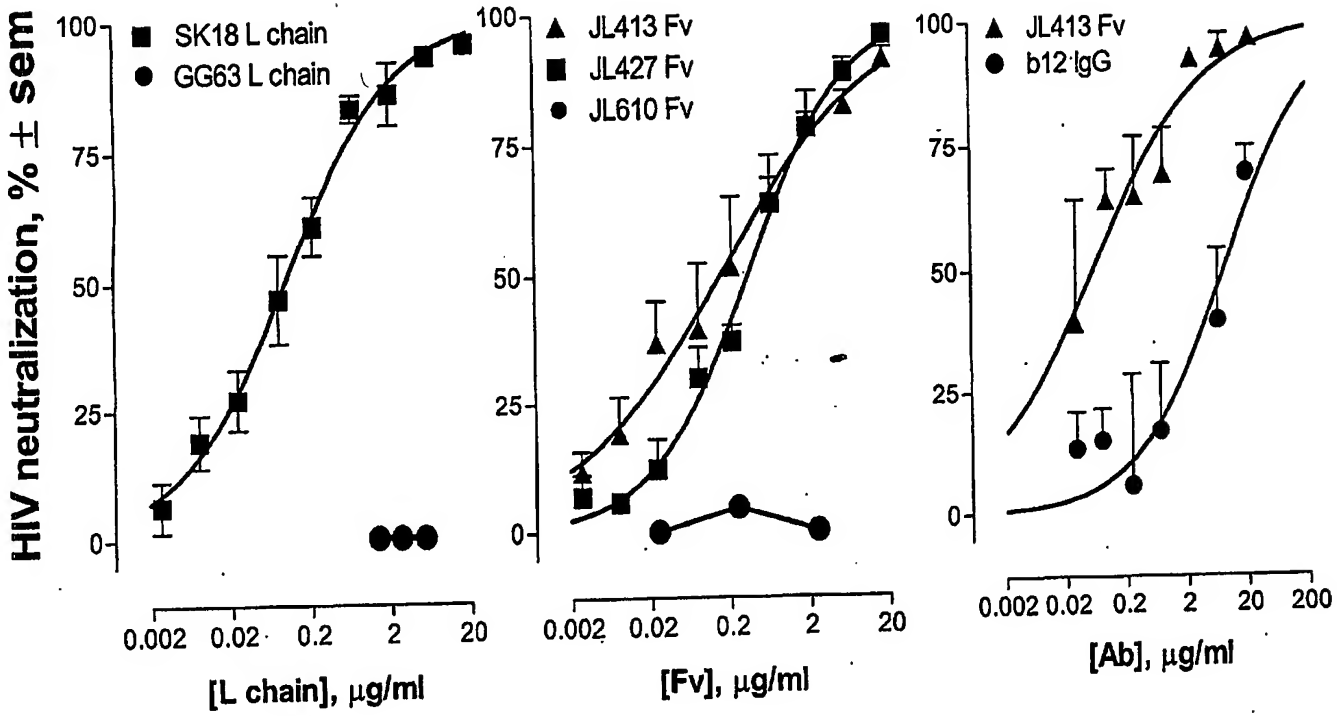

A
B
C
D
A490 ± s.d.
27
[L chain SK18], µg/ml
[Fv JL413], µg/ml
[Fv JL427], µg/ml
Fv JL427
% Inhibition
gp120(421-436)
BSA
Tg
CaM

Figure 3


HIV neutralization, % ± sem
SK18 L chain
GG63 L chain
[L chain], µg/ml
JL413 Fv
JL427 Fv
JL610 Fv
[Fv], µg/ml
JL413 Fv
b12 IgG
[Ab], µg/ml
0
25
50
75
100
0.002
0.02
0.2
2
20
200

Supplementary information, attachment 1: cDNA and deduced amino acid sequences of lupus antibody fragments aligned with their germline V, (D) and J counterparts (Light chain SK18, Fv JL413 and Fv JL427).

Linearly numbered nucleotide (N) and amino acid (R) sequences of lupus V domains shown with no numbering breaks at V, (D), J junctions. Germline counterpart V, (D), J genes are numbered individually, starting with R/N# 1 for each gene. Numbers above and below sequences pertain to lupus clones and germline counterparts, respectively. Overlined (lupus V domains) and underlined (germline counterparts) sequences are complementary determining regions (CDRs). Fv JL413 CDR3H assignment is ambiguous (dotted overline; see alternate alignments 1 and 2, pages 4 and 5). Dots and dashes represent nucleotide and amino acid identities, respectively. Solid vertical lines demarcate V, D and J genes. V gene residues analyzed in Table 1 are C terminal to the broken vertical lines. At the C termini of light chain and the Fv clones are located the complete CL domain and CH1 residues and the N terminal 9 residues of CH1, respectively, followed by the his6 and c-myc tags (HHHHHHGAAEQKLISEEDLN; catcatcatcaccatcacggggccgcagaacaaaaactcatctcagaagaggatctgaat). A linker sequence links the C terminus of the Fv VL domain to the N terminus of the VH domain (SSGGGSGGGGSGGSA; tcgagtggtggaggcggttcaggcggaggtggctctggcggtagtgca). See Fv JL413 VH for discussion of alternate junctional regions aligments.

(A) Light chain SK18 VL domain

R#	1																				20
N#	1																				60
SK18	ASP	ILE	GLN	MET	THR	GLN	SER	PRO	SER	SER	LEU	SER	ALA	SER	VAL	GLY	ASP	ARG	VAL	THR	
SK18	gac	atc	cag	atg	acc	cag	tct	cca	tcc	tcc	ctg	tct	gca	tct	gtg	gga	gac	aga	gtc	acc	
02/012	...	...	...	...	...	...	...	...	...	...	...	...	...	...	..a	...	...	...	...	...	
02/012	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	
N#	1							V gene →													60
R#	1																				20

R#	21																				40
N#	61			CDR1																	120
SK18	VAL	THR	CYS	ARG	ALA	SER	GLN	SER	ILE	SER	SER	TYR	LEU	ASN	TRP	TYR	GLN	GLN	GLN	PRO	
SK18	gtc	act	tgc	cgg	gca	agt	cag	agc	att	agc	agc	tat	tta	aat	tgg	tat	cag	cag	caa	cca	
02/012	a..	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	a..	...	
02/012	ILE	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	LYS	---	
N#	61																				120
R#	21																				40

R#	41																				60
N#	121									CDR2								180			
SK18	GLY	LYS	ALA	PRO	LYS	LEU	LEU	ILE	TYR	ALA	ALA	SER	SER	LEU	GLN	SER	GLY	VAL	PRO	SER	
SK18	ggg	aag	gcc	cct	aaa	ctc	ctg	atc	tat	gct	gca	tcc	agt	ttg	caa	agt	ggg	gtc	cca	tca	
02/012	...	..a	...	...	..g	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
02/012	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	
N#	121																				180
R#	41																				60

R#	61																			80
N#	181																			240
SK18	ARG	PHE	SER	GLY	SER	GLY	SER	GLY	THR	ASP	PHE	THR	LEU	THR	ILE	SER	SER	LEU	GLN	PRO
SK18	agg	ttc	agt	ggc	agt	gga	tct	ggg	aca	gat	ttc	act	ctc	acc	atc	agc	agt	ctg	caa	cct
02/012	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
02/012	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
N#	181																			240
R#	61																			80

											CDR3						
R#	81														95		
N#	241														285		
SK18	GLU	ASP	PHE	ALA	THR	TYR	PHE	CYS	GLN	GLN	SER	TYR	SER	ILE	PRO	*dd*	*d*
SK18	gaa	gat	ttt	gca	act	tac	ttc	tgt	caa	cag	agt	tac	agt	atc	cct	*dd*	*d*
02/012	...	...	...	...	...	...	.a.	...	...	...	...	...	...	.c.	...	cc	g
02/012	---	---	---	---	---	---	TYR	---	---	---	---	---	---	THR	---	PRO	
N#	241														285		
R#	81														95		

← V gene | J gene →

R#		96										107	
N#		286										321	
SK18	*ddd*	ARG	THR	PHE	GLY	GLN	GLY	THR	LYS	VAL	GLU	ILE	LYS	*d*
SK18	*ddd*	cga	acg	ttc	ggc	caa	ggg	acc	aag	gtg	gaa	atc	aaa	*d*
Jk1	tgg	.cg	...	...	...	...	...	...	...	...	...	...	...	c
Jk1	TRP	PRO	---	---	---	---	---	---	---	---	---	---	---	
N#		5										40	
R#		2										13	

J gene →

(B) Fv JL413

VL domain

```
R#          1                                                                                                   20
N#          1                                                                                                   60
JL413 VL    ASP VAL VAL MET THR GLN SER:PRO SER SER VAL SER ALA SER VAL GLY ASP ARG VAL THR
JL413 VL    gat gtt gtg atg act cag tct:cca tct tcc gtg tct gca tct gta gga gac aga gtc acc
L5          ..c a.c ca. ... ..c ... ...:... ... ... ... ... ... ... ... ... ... ... ... ...
L5          --- ILE GLN --- --- --- ---:--- --- --- --- --- --- --- --- --- --- --- --- ---
N#          1                          : V gene →                                           60
R#          1                                                                               20

R#          21                                                                                  40
N#          61                                  CDR1                                                    120
JL413 VL    ILE THR CYS ARG ALA SER GLN GLY ILE GLY ASN TRP LEU ALA TRP TYR GLN GLN LYS PRO
JL413 VL    atc act tgt cgg gcg agt cag ggt att ggc aac tgg tta gcc tgg tat cag cag aaa cct
L5          ... ... ... ... ... ... ... ... ... a.. .g. ... ... ... ... ... ... ... ... ..a
L5          --- --- --- --- --- --- --- --- --- SER SER --- --- --- --- --- --- --- --- ---
N#          61                                                                          120
R#          21                                                                          40

R#          41                                                                                  60
N#          121                                         CDR2                                            180
JL413 VL    GLY LYS ALA HIS ASN LEU LEU ILE TYR GLY ALA SER SER LEU GLN SER GLY VAL PRO SER
JL413 VL    ggg aaa gcc cat aac ctc ctg atc tat ggt gca tcc agt ttg caa agt ggg gtc cca tca
L5          ... ... ... .c. ..g ... ... ... ... .c. ... ... ... ... ... ... ... ... ... ...
L5          --- --- --- PRO LYS --- --- --- --- ALA --- --- --- --- --- --- --- --- --- ---
N#          121                                                                             180
R#          41                                                                          60

R#          61                                                                                  80
N#          181                                                                                     240
JL413 VL    ARG PHE SER GLY SER GLY SER GLY THR ASP PHE THR LEU THR ILE SER SER LEU GLN PRO
JL413 VL    agg ttc agc ggc agt gga tct ggg aca gac ttc act ctc acc atc agc agc ctg cag cct
L5          ... ... ... ... ... ... ... ... ... ..t ... ... ... ... ... ... ... ... ... ...
L5          --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
N#          181                                                                             240
R#          61                                                                          80

R#          81                                                  94
N#          241                                   CDR3          282
JL413 VL    GLU ASP SER ALA THR TYR TYR CYS GLN GLN ALA LEU VAL GLY ddd dd | d
JL413 VL    gaa gac tct gca act tac tat tgt caa cag gct ctc gtg ggt ddd dd | d
L5          ... ..t .t. ... ... ... ... ... ... ... ... ... aa. agt ttc cct cc | g
L5          --- --- PHE --- --- --- --- --- --- --- --- --- ASN SER PHE PRO   PRO
N#          241                                                         282
R#          81                                                  94
                                                                ← V gene | J gene →
```

[redacted]

R#			95								105	
N#			283								315	
JL413 VL		***ddd***	THR	PHE	GLY	GLY	GLY	THR	LYS	VAL	GLU	ILE	LYS	ARG
JL413 VL		***ddd***	act	ttc	ggc	gga	ggg	acc	aag	gtg	gag	atc	aaa	cgc
Jk4		ctc	...	...	...	...	...	...	...	...	...	...	...	***.dd***
Jk4		LEU	---	---	---	---	---	---	---	---	---	---	---	***-dd***
N#			5										37	
R#			2										12	
		J gene→												

Fv JL413 VH domain, alignment 1

```
R#          1                                                                                                    20
N#          1                                                                                                    60
JL413 VH    GLN VAL ASN LEU ARG GLU SER GLY PRO GLY LEU VAL LYS PRO SER GLU THR LEU SER LEU
JL413 VH    cag gtc aac tta agg gag tct ggc cca gga ctg gtg aag cct tcg gag acc ctg tcc ctc
VH4-59      ... ..g c.g .t. ca. ... ..g ... ... ... ... ... ... ... ... ... ... ... ... ...
VH4-59      --- --- GLN --- GLN --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
N#          1                           V gene →                                                                 60
R#          1                                                                                                    20

R#          21                                                                                                   40
N#          61                                                                                                   120
                                                          CDR1
JL413 VH    THR CYS THR VAL SER GLY GLY PHE ILE SER SER TYR TYR TRP SER TRP ILE ARG GLN PRO
JL413 VH    acc tgc act gtc tct ggt ggc ttc atc agt agt tac tac tgg agc tgg atc cgg cag ccc
VH4-59      ... ... ... ... ... ... ... .c. ... ... ... ... ... ... ... ... ... ... ... ...
VH4-59      --- --- --- --- --- --- --- SER --- --- --- --- --- --- --- --- --- --- --- ---
N#          61                                                                                                   120
R#          21                                                                                                   40

R#          41                                                                                                   60
N#          121                                                                                                  180
                                                                                CDR2
JL413 VH    PRO GLY LYS GLY LEU GLU TRP ILE GLY PHE THR TYR TYR SER GLY SER THR TYR TYR ASN
JL413 VH    cca ggg aag gga ctg gag tgg att ggg ttt acc tat tac agt ggg agc acc tac tac aac
VH4-59      ... ... ... ... ... ... ... ... ... .a. .t. ... ... ... ... ... ... a.. ... ...
VH4-59      --- --- --- --- --- --- --- --- --- TYR ILE --- --- --- --- --- --- ASN --- ---
N#          121                                                                                                  180
R#          41                                                                  60

R#          61                                                                                                   80
N#          181                                                                                                  240
            PRO SER LEU LYS SER
JL413 VH    PRO SER LEU LYS SER ARG VAL THR ILE SER VAL ASP THR SER LYS ASN GLN PHE SER LEU
JL413 VH    ccg tcc ctc aag agt cga gtt acc ata tca gta gac acg tct aag aac cag ttc tcc ctg
VH4-59      ..c ... ... ... ... ... ..c ... ... ... ... ... ... ..c ... ... ... ... ... ...
VH4-59      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
N#          181                                                                                                  240
R#          61                                                                  80

R#          81                                  91
N#          241                                 273
JL413 VH    LYS LEU SER SER VAL THR ALA ALA ASP THR ALA ddd ddd ddd ddd ddd ddd dd | d
JL413 VH    aag ctg agc tct gtg acc gct gcg gac acg gcc ddd ddd ddd ddd ddd ddd dd | d
VH4-59      ... ... ... ... ... ... ... ... ... ... ... gtg tat tac tgt gcg aga ga | a
VH4-59      --- --- --- --- --- --- --- --- --- --- --- VAL TYR TYR CYS ALA ARG GLU
N#          241                                 273
R#          81                                  91
                                                ← V gene | D gene →
```

R#						92			96		
N#						274		CDR3	288		
JL413 VH	*ddd*	*ddd*	*ddd*	*ddd*	*ddd*	MET	TYR	CYS	TYR	CYS	
JL413 VH	*ddd*	*ddd*	*ddd*	*ddd*	*ddd*	atg	tat	tgc	tac	tgc	
D2-15	gga	tat	tgt	agt	ggt	ggt	agc	...	...	.c.	
D2-15	GLY	TYR	CYS	SER	GLY	GLY	SER	---	---	SER	
N#						17			31		
R#						6			10		

D gene →

R#							97									103
N#							289							321		
JL413 VH	*dd*	*ddd*	*ddd*	*ddd*	*ddd*	*ddd*	TRP	GLY	GLN	GLY	THR	LEU	VAL	THR	VAL	SER	SER
JL413 VH	*dd*	*ddd*	*ddd*	*ddd*	*ddd*	*ddd*	tgg	ggc	cag	gga	acc	ctg	gtc	acc	gtc	tcc	tca
JH5	ac	aac	tgg	ttc	gac	tcc	...	...	..a	...	...	...	...	...	...	...	...
JH5		ASN	TYR	PHE	ASP	SER	---	---	---	---	---	---	---	---	---	---	---
N#							18									50	
R#							6									16	

J gene →

Fv JL413 VH domain, alignment 2 (junctional region aligned according to referee suggestion)

```
R#          81                                                                  96
N#          241                                                                 288
JL413 VH    LYS LEU SER SER VAL THR ALA ALA ASP THR ALA MET TYR CYS TYR CYS ddd dd | d
JL413 VH    aag ctg agc tct gtg acc gct gcg gac acg gcc atg tat tgc tac tgc ddd dd | d
VH4-59      ... ... ... ... ... ... ... ... ... ... ... g.. ... .a. .gt gcg aga ga | a
VH4-59      --- --- --- --- --- --- --- --- --- --- --- VAL --- TYR CYS ALA ARG   GLU
N#          241                                         273                       |
R#          81                                    91                            |
                                                                  ← V gene        | D gene →

JL413 VH    ddd ddd ddd ddd ddd ddd ddd ddd ddd ddd |
JL413 VH    ddd ddd ddd ddd ddd ddd ddd ddd ddd ddd |
D2-15       gga tat tgt agt ggt ggt agc tgc tac .c.  |
D2-15       GLY TYR CYS SER GLY GLY SER CYS TYR SER  |
N#          2                                   31  |
R#          1                                   10  |
            D gene →

R#                                  97                                          103
N#                                  289                         321
JL413 VH    dd ddd ddd ddd ddd ddd TRP GLY GLN GLY THR LEU VAL THR VAL SER SER
JL413 VH    dd ddd ddd ddd ddd ddd tgg ggc cag gga acc ctg gtc acc gtc tcc tca
JH5         ac aac tgg ttc gac tcc ... ... ..a ... ... ... ... ... ... ... ...
JH5            ASN TYR PHE ASP SER --- --- --- --- --- --- --- --- --- --- ---
N#                                  18                                      50
R#                                  6                                       16
            J gene →
```

Alignment	Nucleotides, #274-288		Amino acids, #91-96	
	Replacements	Deletions	Replacements	Deletions
1	7/15	53	3/5	17
2	7/15	53	4/5	17

(C) Fv JL427

VL domain

```
                                                                                               20
R#        1                                                                                    60
N#        1                                  :                                                 
JL427 VL  GLN SER VAL LEU THR GLN PRO:PRO SER VAL SER GLY ALA PRO GLY GLN ARG VAL THR ILE
JL427 VL  cag tct gtg ttg acg cag ccg:ccc tca gtg tct ggg gcc cca ggg cag agg gtc acc att
V1-17     ... ... ... c.. ..t ... ..a:... ... .c. ... ... a.. ..c ... ... ... ... ... ..c
V1-17     --- --- --- --- --- --- ---:--- --- ALA --- --- THR --- --- --- --- --- --- ---
                                     :                                                 60
N#        1                          : V gene →                                        20
R#        1

                                                                                   40
R#        21                                                                   120
N#        61                     CDR1
JL427 VL  SER CYS SER GLY SER SER SER ASN PHE GLY LEU ASN TYR VAL TYR TRP TYR GLN HIS PHE
JL427 VL  tct tgt tct gga agc agc tcc aac ttc ggc ctt aat tat gta tac tgg tat cag cac ttc
V1-17     ... ... ... ... ... ... ... ... a.. ..a ag. ... ... ... ... ... ..c ... ..g c..
V1-17     --- --- --- --- --- --- --- --- ILE --- SER --- --- --- --- --- --- --- GLN LEU
                                                                                   120
N#        61                                                                       40
R#        21

                                                                                   80
R#        41                                                                           180
N#        121                                       CDR2
JL427 VL  PRO GLY THR ALA PRO LYS LEU LEU ILE TYR ARG ASN ASP GLN ARG PRO LEU GLY VAL PRO
JL427 VL  cca gga acg gcc ccc aaa ctc ctc atc tat agg aat gat cag cgg ccc tta ggg gtc cct
V1-17     ... ... ... ... ... ... ... ... ... ... ..t ... a.. ... ... ... .c. ... ... ...
V1-17     --- --- --- --- --- --- --- --- --- --- SER --- ASN --- --- --- SER --- --- ---
                                                                                       180
N#        121                                                                      80
R#        41

                                                                                   100
R#        81                                                                           240
N#        181
JL427 VL  ALA ARG PHE SER GLY SER LYS SER GLY THR SER ALA SER LEU ALA ILE SER GLY LEU ARG
JL427 VL  gcc cga ttc tct ggc tcc aaa tct ggc acc tca gcc tcc ctg gcc atc agt ggg ctc cgg
V1-17     .a. ... ... ... ... ... ..g ... ... ... ... ... ... ... ... ... ... ... ... ...
V1-17     ASP --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                                                                                       240
N#        181                                                                      100
R#        81

                                                                               120
R#        101                                            CDR3      300
N#        241
JL427 VL  SER GLU ASP GLU ALA ASP TYR TYR CYS GLN SER TYR ASP ASN SER LEU SER GLY dd | d
JL427 VL  tcc gag gat gag gct gat tat tac tgc cag tcc tat gac aac agc ctg agt ggc dd | d
V1-17     ... ... ... ... ... ... ... ... ..t gca g.a .gg ..t g.. ... ... ... ..t cc | t
V1-17     --- --- --- --- --- --- --- --- --- ALA ALA TRP --- ASP --- --- --- ---   PRO
                                                                               300 |
N#        241                                                              120     |
R#        101                                                       ← V gene   |  J gene →
```

```
R#          121                                                 133
N#          241                                                 279
JL427 VL    TRP VAL PHE GLY GLY GLY TYR GLN LEU TYR VAL LEU GLY
JL427 VL    tgg gtg ttc ggc gga ggg acc cag ctc acc gtt tta ggc
JL3         tgg gtg ... ... ... ... ... a.. ..g ... ..c c.. ..t
JL3         --- --- --- --- --- --- --- LYS --- --- --- --- ---
N#          2                                                   40
R#          1                                                   13
            J gene →
```

Fv JL427 VH domain

```
R#          1                                                                                                                   20
N#          1                                                                                                                   60
JL427 VH    GLN VAL GLN LEU GLN GLN SER:GLY GLY GLY LEU VAL GLN PRO GLY ARG SER LEU ARG LEU
JL427 VH    cag gta cag ctg cag cag tca:ggg gga ggc ttg gta cag cct ggg agg tcc ctg aga ctc
VH3-48      g.. ..g ... ... gt. g.. ..t:... ... ... ... ... ... ... ... g.. ... ... ... ...
VH3-48      GLU --- --- --- VAL GLU ---:--- --- --- --- --- --- --- --- GLY --- --- --- ---
N#          1                          : V gene →                                                                               60
R#          1                                                                                                                   20

R#          21                                                                                                          40
N#          61                                        CDR1                                                    120
JL427 VH    SER CYS ALA ALA SER GLY PHE THR PHE SER SER TYR GLY MET HIS TRP VAL ARG GLN ALA
JL427 VH    tcc tgt gca gcg tct gga ttc acc ttc agt agc tat ggc atg cac tgg gtc cgc cag gct
VH3-48      ... ... ... ..c ... ... ... ... ... ... ... ... a.. ... a.. ... ... ... ... ...
VH3-48      --- --- --- --- --- --- --- --- --- --- --- --- SER --- ASN --- --- --- --- ---
N#          61                                                                      120
R#          21                                                                          40

R#          41                                                                                                          60
N#          121                                                                                 CDR2                                180
JL427 VH    PRO GLY LYS GLY LEU GLU TRP VAL SER TYR ILE GLY ARG SER GLY SER HIS THR ASN TYR
JL427 VH    cca ggg aag ggg ctg gag tgg gtt tca tac att ggt aga agt ggt agt cac aca aac tac
VH3-48      ... ... ... ... ... ... ... ... ... ... ... ... a.. ..t ... a.. ... ac. .t. t.. ...
VH3-48      --- --- --- --- --- --- --- --- --- --- --- --- SER SER --- SER --- THR ILE TYR ---
N#          121                                                                                                             180
R#          41                                                                                                          60

R#          61                                                                                                          80
N#          181                                                                                                             240
JL427 VH    ALA ASP SER VAL LYS GLY ARG PHE THR ILE SER ARG ASP ASN SER LYS ASN THR LEU TYR
JL427 VH    gca gac tcc gtg aag ggc cga ttc acc atc tcc aga gac aat tcc aag aac acg ctg tat
VH3-48      ... ... ..t ... ... ... ... ... ... ... ... ... ... ... ... g.. ... ... t.a ... ...
VH3-48      --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ALA --- --- SER --- ---
N#          181                                                                                                             240
R#          61                                                                                                          80

R#          81                                                                                                  98
N#          241                                                                                                         294
JL427 VH    LEU GLN ILE ASN SER LEU ARG ALA GLU ASP THR ALA VAL TYR TYR CYS ALA ARG dd d
JL427 VH    ctg caa ata aac agc ctg aga gcc gag gac acg gct gtg tat tac tgt gcg aga dd d
VH3-48      ... ... ..g ... ... ... ... .a. ... ... ... ... ... ... ... ... ... ... ga t
VH3-48      --- --- MET --- --- --- --- ASP --- --- --- --- --- --- --- --- --- --- --- ASP
N#          241                                                                                                         294
R#          81                                                                                                  98
                                                                                                  ← V gene  J gene →
```

```
JL427 VH   d ddd ddd ddd
JL427 VH   d ddd ddd ddd
D7-27      t aac tgg gga
D7-27        THR TRP GLY
N#           3       11
R#           1       3
           D gene →
           /
R#         99                                                                  118
N#         294          CDR3                                                   353
JL427 VH   GLY LEU PRO ASN TYR GLY MET ASP ILE TRP GLY GLN GLY THR THR VAL THR VAL SER SER
JL427 VH   ggg ctg cca aac tac ggt atg gac atc tgg ggc caa ggg acc acg gtc acc gtc tcc tca
JH6        .ac tac tac tac ... ... ... ... g.. ... ... ... ... ... ... ... ... ... ... ...
JH6        ASP TYR TYR TYR --- --- --- --- VAL --- --- --- --- --- --- --- --- --- --- ---
N#         1                                                                       60
R#         1                                                                       20
           J gene →
```

Supplementary information, attachment 2: Amino acid sequences of twelve VH domains of Fv clones isolated form the lupus phage library. Clones 78 and 129 isolated by binding to immobilized prothrombin (unpublished data). Clones SP7D2, SP1A2 and SP8C5 isolated by binding to immobilized extracellular domain of epidermal growth factor receptor (Genbank accession# AF329456, AF329457, AF329458, respectively; Planque et al. FASEB J. 17, 136-143 (2003)). Clones DM319, DM412, DM408, DM506, JB14, JB43 and JB48 identified by binding to vasoactive intestinal polypeptide (Genbank accession# AF416909, AF416907, AF509586, AF509587, AF416909, AF416910, AF416908, respectively; Bangale et al Peptides 23, 2251-2257 (2002); Bangale et al FASEB J. *In press*, attached manuscript 3). No truncation of FR3 is evident in any clones. Unambiguous CDR3 segments ranging in length from 9 - 18 amino acids are evident in the clones.

Fv 78
QLVESGGGLVQPGGSLRLSCAASGFTFDDYAMHWVRQAPGKGLEWVSGISWNSGSIGYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKDLSSGWIPPRKWYYGMDXWGQGTTVTVSS

Fv 129
QLQESGGGLVQPGRSLRLSCAASGFAFSSYAMHWVRQAPGKGLEWVAVISYDGSNKYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCARDLYYSKYYYYGMDVWGQGTTVTVSS

Fv SP7D2
LQVQLQESGPGLVKPSQTLSLTCTVSGGSISSGGYYWSWIRQHPGKGLEWIGYIYYSGSTYYNPSLKSRVTISVDKSKNQFSLKLSSVTAADTAVYYCARASGYSYGGDFDYWGQGTLVTVSS

Fv SP1A2
LQVQLQQSGAEVKKPGESLKISCKGSGYSFTSYWIGWVRQMPGKGLEWMGIIYPGDSDTRYSPSFQGQVTISADKSISTAYLQWSSLKASDTAMYYCARHRAASGSPDACDYWGQGTLVTVSS

Fv SP8C5
LQVQLVQSGAEVKKPGSSVKVSCKASGGTFSSNAISWVRQAPGQGFEWMGWINPYTGDTNYAQKFQGWVTMTRDTSISTAYMELSRLTSDDTAVYYCAIPLGLRRAFDIWGQGTMVTVSS

Fv DM319
LQVQLLQSAAEVKKPGTSVRVSCRSSGYTFTSFYMHWVRQAPGQRLEWMGMINPKNGVTNYAQKFKGRVTMTRDTSTTTVYMELSRLRSEDRALYYCACLFGGGYDAMDVWGQGTTVTVSS

Fv DM412
LQVQLQESGPGLVKPSETLSLTCTVSGGSISSSSYYWSWIRQHPGKGLEWIGYIYYSGSTNYNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARFGGSGWNDYWGQGTLVTVSS

Fv DM408
LEVQLVESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKMYSSGWYFDYWGQGTLVTVSS

Fv DM506
LQVQLQESGPGLVKPSQTLSLTCTVSGGSISSGGYYWGWIRQPPGKGLEWIGSIYYSGSTYYNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARNDDFWSGYYGYWGQGTLVTVSS

Fv JB14
LQVQLQQSGPRLVKPSGTLSLTCTVSGGSISSSSYYWGWIRQHPGKGLEWIGYIYYSGSTYYNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARLOGARGYSYGYGIDYWGQGTLVTVSS

Fv JB43
LQVQLQESGPGLVKPSETLSLTCTVSGGSVSSGSYYWSWIRQPAGKGLEWIGRIYTSGSTNYNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARVGSGWYGGYYYGMDVWGQGTTVTVSS

Fv JB48
LQVQLQQSGAEMKRPGASVKVSCKASGGTFSGYAINWMRQAPGQRLEWIGWIVVSGGHAAYAQNFQGRVTMTRDTSISTAYMELSRLRSDDTAVYYCAIPLGLRRAFDIWGQGTMVTVSS